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Docket No.: 56238 (71526)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT:	M. Sakanaka, et al.	EXAMINER:	D. Khare
SERIAL NO.:	09/913,669	GROUP:	1623
FILED:	August 16, 2001		
FOR:	CEREBROVASCULAR REGENERATION/ RECONSTRUCTION-PROMOTING AGENTS AND SECONDARY NERVOUS TISSUE DEGENERATION-INHIBITING AGENTS COMPRISING GINSENOSIDE RB1		

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AMENDMENT AND RESPONSE

Sir:

Applicants are in receipt of the Office Action dated June 1, 2005 and request reconsideration of the above-identified application in view of the following amendments and remarks. Please amend the above-identified application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 5 of this paper.

Amendments To The Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of claims:

1-31. (Cancelled).

32. (Currently Amended) A method of treating a patient suffering from a ~~for the treatment of diseases caused by~~ traumatic or compression ~~injuries to~~ injury of a nervous tissue~~tissues~~, the method ~~comprising~~ comprises the step of administering to a patient a therapeutically effective amount of a pharmaceutical composition comprising a therapeutic agent selected from ginsenoside Rb_1, its metabolites and salts thereof.

33. (Currently Amended) The method of claim 32, wherein the therapeutically effective amount of the pharmaceutical composition ~~is capable of suppressing the~~ suppresses secondary degeneration of the injured nervous tissue ~~tissues caused by traumatic or compression injuries to the nervous tissues~~.

34. (Currently Amended) The method of claim 33, wherein the nervous ~~tissues are spinal cords~~tissue is a spinal cord.

35. (Currently Amended) The method of claim 32, wherein the therapeutically effective amount of the pharmaceutical composition ameliorates ~~agent is capable ameliorating~~ paralysis or paraplegia caused by ~~traumatic or compression injuries~~ the injury to the nervous ~~tissues~~tissue.

36. (Currently Amended) The method of claim 35, wherein the nervous ~~tissues are spinal cords~~tissue is a spinal cord.

37. (Cancelled).

38 (Currently Amended) The method of claim 32, wherein the therapeutically effective amount of the pharmaceutical composition ~~is capable of suppressing the~~ suppresses secondary degeneration ~~of the nervous tissues~~ caused by demyelination.

39. (Currently Amended) The method of claim 38, wherein the therapeutically effective amount of the pharmaceutical composition ~~is capable of suppressing~~ suppresses apoptosis or apoptosis-like cell death of oligodendrocytes.

40. (Previously Presented) The method of claim 32, wherein the pharmaceutical composition is administered intravenously to the patient.

41. (Previously Presented) The method of claim 32, wherein the pharmaceutical composition is administered in a single intravenous infusion or in a continuous intravenous infusion to the patient.

42. (Currently Amended) The method of claim 32, wherein the therapeutically effective amount of the pharmaceutical composition administered to the patient provides a concentration of 1 ng/ml or less of ginsenoside Rb_1, its metabolites or salts thereof ~~to~~ in the extracellular fluid ~~in~~ of a lesion ~~at a concentration of 1 ng/ml or less~~.

43. (Currently Amended) The method of claim 32, wherein the therapeutically effective amount of the pharmaceutical composition administered to the patient provides a concentration of 1 pg/ml or less of ginsenoside Rb_1, its metabolites or salts thereof ~~to~~in the extracellular fluid ~~in~~ of a lesion ~~at a concentration of 1 pg/ml or less~~.

44. (Currently Amendede) The method of claim 32, wherein the therapeutically effective amount of the pharmaceutical composition administered to the patient provides a concentration of 100 fg/ml or less of ginsenoside Rb_1, its metabolites or salts thereof ~~to~~ in the extracellular fluid ~~in~~ of a lesion ~~at a concentration of 100 fg/ml or less~~.

45-51. (Cancelled).

52. (Currently Amended) The method of claim 32, wherein the ~~diseases caused by~~ traumatic or compression injuries to the nervous tissue of the patients ~~comprise~~ cause spinal cord injury, head injury, neurotrauma, cerebral hemorrhage, subarachnoid hemorrhage, peripheral nerve injuries, Parkinson's Disease, demyelinating diseases, retinal detachment, herniation of intervertebral disks, spinal canal stenosis, spondylolysis, slipping diseases, cervical spondylolysis, ossification of the posterior longitudinal ligament or facial nerve paralysis.

53. (Previously Presented) The method of claim 32, wherein the nervous tissues are spinal cords.

REMARKS

The Applicants appreciate the Examiner's thorough examination of the subject application. Applicants request reconsideration of the subject application based on the following remarks.

Claims 32-36, 38-44, and 52-53 are pending in the application. Claims 1-31, 37, and 45-51 have been cancelled. Claims 32-36, 38, 39, 42, and 52 have been amended to more clearly define the invention. Support can be found at least in the claims as originally filed. No new matter has been introduced by the instant amendments.

Claims 32-36, 38-44 and 52-53 were rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention of record. It is further alleged that the specification does not reasonably provide enablement for the treatment of diseases caused by injuries to nervous tissues or the spinal cord.

The Office Action misinterprets the subject matter of the invention. At page 2, line 6 of the last paragraph, the Office Action states that "the specification, while enabling a method of inducing apoptosis or apoptosis-like cell death of oligodendrocytes comprising administering an effective amount of a pharmaceutical composition comprising a therapeutic agent ginsenoside Rb_1, ..."

The claimed invention provides methods of treating traumatic and/or compressive injuries to nervous tissue of a patient by administering a therapeutically effective amount of a pharmaceutical composition comprising a ginsenoside Rb_1, its metabolites or salt thereof. The dosage of ginsenoside ameliorates the damage caused by the injury by ***preventing*** cell death in the injured nervous tissue.

Enabling disclosure supporting the instant claims can be found throughout the spefication. In particular, the specification provides preferred dosage amounts to obtain the desired therapeutic effect in human patients. See, for example page 30, line 17 to page 31, line 10 which provide that a therapeutically effective amount of the ginsenoside Rb_1 for human patients with cerebral apoplexy is between about 1.2 mg/day to 12 mg/day for a 60 kg human patient. Suitable therapeutically effective amounts for other diseases or disorders caused by damage to nervous tissues can be readily calculated from rat models and/or the dosages for cerebral apoplexy.

Under the heading titled "Response to Arguments" the Examiner averred that the specification is limited to the effect of ginsenoside Rb_1 on rats with spinal cord injuries. Applicants respectfully disagree. Rats are known in the art to successfully model human therapeutic applications for spinal cord injuries. See, for example, D. L. Behrmann, et al., "Modeling of Acute Spinal Cord Injury in the Rat: Neuroproteciton and Enhanced Recovery with Methylperdnisolone, U-74006F and YM014673" *Experimental Neurology*, **126**, 61-75 (1994), a copy of which is provided in Appendix A.

The Behrmann publication provides an illustrative example of controlled spinal cord injury in rats being used as a model for testing therapeutic agents efficacy in humans. Thus, the instant specification provides ample written description of the claimed invention for one of ordinary skill in the art to understand and practice the instant invention.

Additional support can be found at least in the specification as originally filed, in Example 4, which demonstrates the effect of ginsenoside Rb_1 for the treatment of diseases caused by injuries to the spinal cord (a nervous tissue).

Example 4 demonstrates that anesthetized rats were subjected to spinal cord injury by compression loading. The vertebral arch was removed from the rats to expose

the spinal cord and the spinal cord injury was prepared by directly loading 20 g of compression for 20 min. Because the rats' weight is about 300 g, and 20 g of compression was applied, approximately 1/15 of the rats' weight was administered as the compression loading. This weight is comparable to 4 kg (~8.8 pounds) weight being loaded directly onto the exposed spinal cord of a human weighing 60 kg (~132.3 pounds).

The compression applied to the nervous tissues and spinal cord tissues resulted in the destruction of said tissues, and resulting neuroparalysis. Those of ordinary skill in the art are aware that the seriousness of these injuries are comparable to other models of neurotrauma, such as head injuries and brain injuries. Thus, Example 4 demonstrates a model of traumatic or compression injury to the nervous tissues.

When a saline solution was administered as a treatment, it was determined that the rat exhibited paraplegia in both hind limbs (Figure 8A). However, when a solution of ginsenoside Rb_1 was administered, the paraplegia of both hind limbs was significantly ameliorated (Figure 8B). Figure 8B demonstrates that the rat had the ability to stand up with the aid of a standing bar. Additionally, Figure 9 shows that the motor ability of the rats with spinal cord injuries was significantly ameliorated after administration of ginsenoside Rb_1.

Among neurotrauma, those of ordinary skill in the art are aware that spinal cord injuries are among the most difficult to treat. It is therefore determined that effective compounds for the treatment of serious spinal cord injuries can be expected to be effective on general neurotrauma, such as head injuries and peripheral nerve injuries.

Therefore, Applicants respectfully submit that the instant application provides sufficient enablement for the treatment of diseases caused by traumatic or compression injuries to nervous tissues, comprising the step of administering to a patient a therapeutically effective amount of a pharmaceutical composition comprising a therapeutic agent selected from ginsenoside Rb_1, its metabolites and salts thereof.

Applicants indicate that the rejection is thus obviated and respectfully request withdrawal of the rejection.

The Examiner is hereby authorized to charge our deposit account no. 04-1105 should any fee be deemed necessary.

Respectfully submitted,

Date: August 1, 2005

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APPENDIX A

D. L. Behrmann, et al., "Modeling of Acute Spinal Cord Injury in the Rat: Neuroproteciton and Enhanced Recovery with Methylperdnisolone, U-74006F and YM014673" *Experimental Neurology*, **126**, 61-75 (1994)

EXPERIMENTAL NEUROLOGY 126, 61–75 (1994)

Modeling of Acute Spinal Cord Injury in the Rat: Neuroprotection and Enhanced Recovery with Methylprednisolone, U-74006F and YM-14673[1]

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We used a new injury device that produces consistent spinal cord contusion injuries (T8) in rats to compare the behavioral and histologic effects of methylprednisolone sodium succinate (MPSS) administration, the clinical standard of therapy after acute spinal cord injury (ASCI), with the 21-aminosteroid, U-74006F (U74), and the TRH analogue, YM-14673 (YM), at different trauma doses. Three sequential experiments were conducted: *Experiment 1.* U74 (3.0/1.5/1.5 mg/kg; 10/5/5 mg/kg; 30/15/15 mg/kg), MPSS (30/15/15 mg/kg), or vehicle were administered intravenously (iv) at 5 min, 2 and 6 h after the injury (n = 8/group). U74 (10/5/5 mg/kg) and MPSS animals scored better than controls (Days 8–43) in open field walking (OFW); no other differences were seen between groups. *Experiment 2.* Dose–response evaluation of MPSS determined more effective doses. Groups (n = 16) receiving 30/30/30/30 mg/kg and 60/60/60/60 mg/kg iv at 5 min and 2, 4, and 6 h after the injury had better OFW scores than controls (Days 8–29; Day 29). Both groups performed better than controls (Days 8–29) on inclined plane (IP); 30 mg/kg animals scored higher on Day 29. Percentage tissue spared (%TS) at the lesion center was greater for 60 mg/kg animals (23.4%) than controls (17.3%). *Experiment 3.* Compounds were administered as in experiment 2 (n = 15/group); MPSS (60/30/30/30 mg/kg) and YM (1/1/1/1 mg/kg and 1 mg/kg/day ip) were most effective. YM and MPSS combination produced no additive effects. YM animals scored better than MPSS and control animals in OFW (Days 8–29) and better than controls on IP (Days 8–29; Day 29) and grid walking (Day 29). MPSS animals scored better than controls on IP (Days 8–29). YM and MPSS groups had greater %TS than controls.

This series of experiments demonstrates the utility of this injury model and simple behavioral measures for preclinical assessment of pharmacologic agents. Under these experimental conditions, U74 demonstrated equivalent efficacy to MPSS, and YM demonstrated greater efficacy than MPSS in the treatment of ASCI. © 1994 Academic Press, Inc.

[1] This series of experiments was presented in partial fulfillment of the requirements for the Doctor of Philosophy degree in Neuroscience at The Ohio State University.

INTRODUCTION

The recent findings of the Second National Acute Spinal Cord Injury Study (NASCIS 2) indicated that patients given methylprednisolone sodium succinate (MPSS) within 8 h of their injury had improved recovery of neurologic function (21, 22). This study provided clinical support for the concept that secondary injury processes after traumatic injuries of the spinal cord (45, 85, 102) are amenable to treatment and constituted the first effective application of results from preclinical studies to clinical trials (3, 23, 24, 26, 70, 104). Renewed interest in development of more effective therapies has resulted in a number of potential therapeutic agents; however, their examination in clinical trials is limited by practical and ethical considerations. Thus, experimental models that produce consistent injuries that are clinically relevant are necessary for preclinical screening of these compounds (103).

A spinal cord injury device for use in rats has been developed for this purpose at The Ohio State University. This device uses well-controlled differences in brief (approximately 20 ms) displacement of the spinal cord to produce graded, consistent contusion injuries of the thoracic cord; injury severity/trauma dose (spinal cord displacement and force) has been shown to predict lesion size and functional outcome of the animals (10, 17, 31, 73, 74, 80, 82). This model has recently been demonstrated to be effective in discriminating small but clinically relevant behavioral and histologic effects after administration of compounds immediately after injury (14).

We used this injury model to compare the effects of MPSS administration after acute spinal cord injury with that of the 21-aminosteroid, U-74006F, and the TRH analogue, YM-14673. U-74006F, or Tirilazad Mesylate, is a derivative of MPSS that has even greater antioxidant capacity, but lacks any glucocorticoid activity (27, 47, 52). YM-14673 is an N-terminal modified TRH analogue that results in a half-life that is approximately five times longer than that of TRH; YM-14673 is 10–100 times more potent than TRH and produces 8–36 times longer acting *in vivo* central facilitatory effects than

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TRH (92, 93). Both U-74006F (1, 2, 47, 58) and YM-14673 (35, 76) have previously been reported to have protective effects after traumatic spinal cord injuries; U-74006F is currently in clinical trial (NASCIS 3).

Three sequential experiments were performed. In experiment 1, a dose–response of U-74006F was compared with the dose of MPSS currently used to treat clinical spinal cord injury. In experiment 2, a dose–response of MPSS was performed to determine a more effective dose in this injury model. Statistical power analysis was then performed to estimate the number of animals needed in the next experiment to reproduce the effects of MPSS (90). In experiment 3, MPSS therapy was compared with YM-14763. Acute, chronic, and acute/chronic administration of YM-14673 were examined, as well as the effects of combined MPSS and YM-14673 treatment. Preliminary results of these experiments have been reported (12, 13, 32).

MATERIALS AND METHODS

Compounds

Methylprednisolone sodium succinate (Upjohn Co., Kalamazoo, Michigan) was purchased from The Ohio State University Hospital pharmacy. YM-14673, N^{α}-[[(*S*)-4-oxo-2-azetidinyl] carbonyl]-L-histidyl-L-prolineamide dihydrate was generously provided by Dr. Minoru Yamamoto (Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan). U-74006F, 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methylpregna-1,4,9(11)-triene-3,20-dione, monomethane sulfonate was provided as a gift from Dr. Ed Hall (Upjohn Co., Kalamazoo, MI). MPSS and YM-14673 were dissolved in sterile water. U-74006F was dissolved in buffered-sodium citrate solution.

Subjects

Two hundred thirty-three female albino rats (Harlan Sprague–Dawley Inc., Indianapolis, IN; experiment 1, $n = 43$; experiment 2, $n = 83$; experiment 3, $n = 107$) were housed 2 per cage to reduce the incidence of autotomy (18). Animals were maintained in a 12-h light/dark cycle, with water and food freely available.

Surgical Procedures and Postoperative Care

The animals were anesthetized with an intraperitoneal (ip) injection of Ketaset (80 mg/kg; ketamine HCL, Aveco Co., Fort Dodge, IA) and Rompun (10 mg/kg; xylazine, Mobay Corp., Shawnee, KS). Before the procedure, antibiotics were administered subcutaneously as prophylaxis against wound and bladder infections as follows: experiment 1, Durapen (6000 U BID × 24 h; penicillin G benzathine and penicillin G procaine, Vedco, Inc., Overland Park, KS) and Di-trim (0.2 cc qD × 24 h; sulfadiazine 40 mg/cc and trimethoprim 8 mg/cc sc, Syntex Animal Health Inc., West Des Moines, IA); experiments 2 and 3, Durapen (20,000 U BID × 48 h) and Gentocin (0.25 mg qD × 48 h; gentamicin sulfate, Schering Corp., Animal Health Division, Kenilworth, NJ). Lacrilube ophthalmic ointment (Allergan Pharmaceuticals, Irvine, CA) was used to prevent drying of the eyes. The animals' temperatures were monitored and maintained between 36 and 38°C during the procedures and the immediate postoperative period. All surgery was performed using sterile technique.

The rats underwent a complete single level laminectomy at T8. The animals were secured to the injury device frame at the T7 and T9 spinous processes. The tip of the impactor probe (2-mm diameter) was touched to the dorsal surface of the cord (dura intact) with immediate feedback from a force transducer, and the animals were injured by a very brief (approximately 20 ms), predetermined displacement of the cord as previously described (10, 17, 31, 73, 74, 82).

After the injuries, Furazolidone 4% aerosol aseptic powder (Veterinary Products Lab., Phoenix, AZ) was sprayed over the incisions. Lactated Ringer's solution (5 cc) was given subcutaneously; the animals in experiments 2 and 3 received an additional 5 cc on Postoperative Day 1. Additional parenteral fluids were provided as needed during the next 24–48 h to compensate for poor oral intake. Bladders were expressed at least twice a day until adequate spontaneous voiding returned (usually by 10 days). SMZ–TMP (≈0.5 cc; 40 mg/cc sulfamethoxazole and 8 mg/cc trimethoprim oral suspension, Phoenix Pharmaceutical, Inc., St. Joseph, MO) was administered each day as prophylaxis against urinary tract infections until the rats no longer required bladder expression. Cecon oral solution (10 mg; ascorbic acid, Abbott Lab., North Chicago, IL) was also given daily to all subjects throughout the study to acidify the urine and to accelerate wound healing.

For intravenous (iv) drug delivery, each animal's external jugular vein was cannulated with PE-50 polyethylene tubing (i.d. 0.58 mm, o.d. 0.965 mm; Clay Adams, Division of Becton–Dickinson Co., Parsippany, NJ). The tubing was tunneled subcutaneously to the back where it protruded from the inferior aspect of the wound for easy access. The vein was kept patent between drug infusions by attaching the free end of the PE-50 tubing to an Alzet mini-osmotic pump (Model 2ML1; Alza Corp., Palo Alto, CA) that was wrapped in a moist gauze sponge and Parafilm "M" (Laboratory Film, American National Can, Greenwich, CT). After completion of the last bolus infusion, the free end of the PE-50 tubing was sealed by electrocautery and cut so that the end retracted within the subcutaneous space.

The animals were sacrificed by intracardiac perfusion with 10% buffered formalin under deep anesthesia with ketamine–xylazine (80 and 10 mg/kg ip, respectively) on Postoperative Day 43 (experiment 1) or Day 29 (experi-

ments 2 and 3). The spinal cords were removed and stored in formalin until histologic preparation.

Drug Administration

Experiment 1. The bolus doses of U-74006F used in this study were based on the results of a previous dose–response study by Anderson *et al.* (1) that examined neurologic outcome in cats after a spinal cord compression injury. The bolus dose of MPSS was approximated from the effective clinical dose (21).

Compounds or equal volume vehicle (buffered-sodium citrate solution) were administered intravenously at 5 min, 2 and 6 h after the injury. Animals were randomly assigned to one of the following study groups: U-74006F (3.0/1.5/1.5 mg/kg, $n = 8$; 10/5/5 mg/kg, $n = 8$; 30/15/15 mg/kg, $n = 8$), MPSS (30/15/15 mg/kg, $n = 8$), or controls ($n = 8$).

Experiment 2. Based on the results of experiment 1, the acute dose of MPSS used clinically (30/15/15 mg/kg) appeared to be insufficient in this rat model. Therefore, a full dose–response evaluation was performed to determine a more effective dose.

MPSS or an equal volume bacteriostatic water was administered intravenously at 5 min, 2, 4, and 6 h after the injury. The animals were randomly assigned to one of the following study groups in part 1 of this experiment: MPSS (30/15/15/15 mg/kg, $n = 8$; 30/30/30/30 mg/kg, $n = 8$; 45/45/45/45 mg/kg, $n = 8$; 60/60/60/60 mg/kg, $n = 8$) or controls ($n = 8$).

The behavioral results from these groups demonstrated that the highest doses of MPSS were most effective; therefore, in part 2 of this experiment, the dose–response curve was shifted to include even higher doses as follows: MPSS (30/30/30/30 mg/kg, $n = 8$; 60/60/60/60 mg/kg, $n = 8$; 90/90/90/90 mg/kg, $n = 8$; 120/120/120/120 mg/kg, $n = 8$; 300/300/300/300 mg/kg, $n = 2$) and controls ($n = 8$).

Experiment 3. The results of experiment 2 demonstrated that MPSS doses of 30/30/30/30 and 60/60/60/60 mg/kg were equally and maximally effective. As a result, the 30/30/30/30 mg/kg dose was repeated in this experiment, and an additional dose of 60/30/30/30 mg/kg was evaluated. The YM-14673 dose of 1 mg/kg has been shown in a previous dose–response study by Faden (35) to be most effective after experimental spinal cord injury in rats, and confirmed in this lab to have protective effects (14). This same dose was also administered chronically based on the reported neuroprotective actions of YM-14673 when administered 1 week after middle cerebral artery occlusion in rats (100). MPSS was not administered chronically because of potential adverse side effects and its mechanism of action as an inhibitor of lipid peroxidation early after the injury (52).

Compound or an equal volume bacteriostatic water was administered intravenously at 5 min, 2, 4, and 6 h after the injury. Two groups of YM-14673 animals received daily intraperitoneal injections of the compound as indicated. The animals were randomly assigned to one of the following study groups: YM-14673 (acute administration, 1/1/1/1 mg/kg iv, $n = 15$; acute/chronic administration, 1/1/1/1 mg/kg iv and 1 mg/kg/day ip over Days 1–29, $n = 15$; chronic administration, 1 mg/kg/day ip over Days 15–29, $n = 15$), MPSS (acute administration, 30/30/30/30 mg/kg iv, $n = 15$; acute administration, 60/30/30/30 mg/kg iv, $n = 15$), YM-14673/MPSS (acute administration, YM-14673 1/1/1/1 mg/kg iv and MPSS 30/30/30/30 mg/kg iv, $n = 15$), and controls ($n = 15$).

Behavioral Assessment

The animals were trained daily on the respective behavioral measures for 3 weeks before entering the study. Each was tested the week before the injury (Day 0) and subsequently after the injury as follows: experiment 1—open field walking (Days 1, 2, 8, 15, 22, 29, 43), inclined plane (Days 8, 15, 22, 29, 43), grid walking (Days 29, 43), and footprint analysis (Days 29, 43); experiment 2—open field walking (Days 1, 2, 8, 15, 22, 29) and inclined plane (Days 8, 15, 22, 29); experiment 3—open field walking (Days 1, 2, 8, 15, 22, 29), inclined plane (Days 8, 15, 22, 29), and grid walking (Day 29). In each experiment, testing was performed for at least 4 weeks after injury; previous studies have shown that behavioral recovery does not significantly change after 4 weeks (17). All testing in experiment 3 before Day 29 was performed 30 min after either a sham ip injection (bacteriostatic water) or injection of YM-14673 (1 mg/kg, only those groups receiving chronic administration of YM-14673); testing of the animals on Day 29 was performed both before and after all of the animals were injected with YM-14673. This protocol was followed to examine the reported central facilitatory and increased locomotor effects of YM-14673 administration on these behavioral tests (79, 93, 94).

The behavioral tests were performed by the same two examiners who were unaware of the treatment groups. Hindlimbs were assessed independently (except inclined plane) to identify asymmetry in neurologic recovery (42). Minimal differences were noted; therefore, only the average score for both hindlimbs is reported. A more detailed description of these behavioral tests has been previously reported (9, 17, 31).

The open field walking task measured the animal's gross hindlimb locomotor ability during a 5-min testing period. Two animals were observed simultaneously to stimulate locomotor activity and were scored based upon a modified Tarlov scale (84) as shown in Table 1. If agreement between two scores could not be reached for the two examiners (e.g., 3.3 vs 3.7), then the average of the two scores was recorded (3.5).

The inclined plane task was adapted from Rivlin and Tator (77) and measured the animal's ability to main-

tain its body position on an inclined board for 5 s. Animals were tested at 5° intervals for each of three positions: head up, right side up, and left side up; the average score for the three positions was used for all data analysis.

The grid walking test (31) examined the animal's ability to walk over a wire grid (4 × 4-cm spaces) during a 3-min testing period. The rats were required to actively walk for a minimum of 30 s or the trial was repeated. Dividing the total number of footfalls through the grid spaces by the actual locomotion time provided the number of footfalls per second of locomotion. Animals were unable to be tested if they had not recovered sufficient coordinated hindlimb function (open field walking score of 3.3 or better).

The footprint data were derived from a permanent inked record of the animals' footprints as described by others (28, 83). The forepaws and hindpaws were placed in red and black ink, respectively, and the animal walked over a paper strip in a 6-ft. straight alley runway to obtain a food reward. From one to three trials were performed (as necessary) to obtain an adequate number of legible footprints (minimum of five right and left complete stride lengths) with the rat walking at a consistent pace. If, after the third trial, these criteria were not met, the animal was determined to be untestable. As was the case with grid walking, at least some recovery of coordinated locomotor activity is required for meaningful testing. The footprint data were then derived from these recorded footprints (Fig. 1). To obtain the number of front footprints/ft. walked, the total number of front footprints was counted from the best record of footprints (one individual trial) and then divided by the distance walked.

TABLE 1

Open Field Walking Scale

0	No spontaneous movement
1	Movement in the hip and/or knee, but not ankle
	0.7 Slight movement
	1.3 Active movement at hip and knee, none at ankle
2	Movement of the limb in all three major joints
	1.7 Minimal movement at ankle
	2.3 Attempts at support
3	Active support, uncoordinated gait
	2.7 Support in stance only
	3.3 Intermittent bouts of coordinated gait
4	Coordination of forelimbs and hindlimbs in gait
	3.7 Lack of control of ankle or foot, walks on knuckles or on the medial surface of foot
	4.3 Improved hindlimb postural support, abdomen not low to the ground, few toe drags
5	Normal gait and base of support, no loss of balance on fast turns, no toe drags
	4.7 One or two toe drags, slight unsteadiness turning at full speed

Histology

Eighteen-millimeter segments of the formalin fixed spinal cords were embedded in paraffin blocks and serially sectioned transversely at 20-μm intervals. Every fifth section was stained for Nissl substance with cresyl echt violet and for myelin with luxol fast blue. Camera lucida drawings of those sections representative of the lesion centers were made under low-power light microscopy (4× magnification); higher magnification (10×) was used to more readily differentiate residual normal tissue (Fig. 10). The lesions were characterized by areas of cyst formation and cavitation, gliosis, and grossly swollen axons. The white matter was judged to be spared if the myelin staining (bright blue color) and density was grossly normal in appearance, there was a lack of dense gliosis, and relatively few swollen fibers. The gray matter was judged to be spared if neurons and glia in approximately normal densities were present. The amount of spared tissue (normal appearing and stained neurons and myelinated axons) was determined by an investigator (D.L.B.) who was unaware of the treatment groups. Spared tissue was reported as a percentage of the total cross-sectional area of the cord (area spared tissue/total area cord × 100), as previously described (17, 31, 70). At the injury levels used in this study, only small amounts of residual gray matter were present; therefore, spared tissue represents spared white matter.

Data Analysis

The final open field walking scores were analyzed by the Kruskall–Wallis nonparametric analysis of variance, and post hoc comparisons were made using Mann–

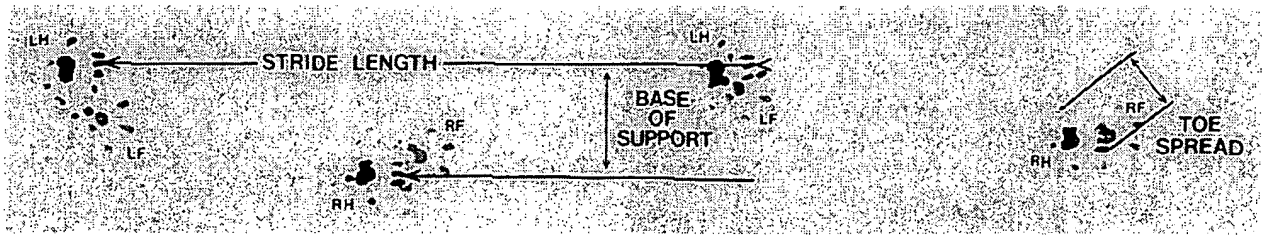

FIG. 1. Forelimb (light gray) and hindlimb (dark gray) footprints of a normal animal, illustrating the stride length, toe spread, and base of support measures. LH, left hindlimb; LF, left forelimb; RH, right hindlimb; RF, right forelimb.

Whitney U tests. All other final behavioral scores, injury parameters, weights, and histologic results were evaluated by one-factor analysis of variance and Duncan's post hoc comparisons (90). Analysis of the behavioral recovery patterns over days tested was performed by multiple-factor repeated measures analysis of variance and Duncan's post hoc comparisons (an equivalent statistical measure for nonparametric data does not exist and therefore this method was used as a close approximation for the open field walking data).

A two sample ($k = 2$) power analysis (90) was performed in experiment 2 to evaluate the number of animals required to reproduce (in experiment 3) the behavioral and histologic effects of MPSS treatment. The average increase in the behavioral and histologic scores (compared with the controls) for the 30 and 60 mg/kg MPSS groups, and powers (p = 1-beta) of $p = 0.90$ and $p = 0.70$ at an alpha = 0.05, were used to estimate the sample size (Table 4).

An alpha of less than 0.05 was chosen as the significance level for all statistical analysis. Unless otherwise indicated, all data are presented as the mean ± SEM. All data analyses were performed with Statgraphics Statistical Graphics System (Version 5.0).

RESULTS

Experiment 1

Subjects. In general, the animals remained in good health throughout the study. The mean weight of the animals ($n = 40$) at the time of their injuries (Day 0) was 272.3 ± 2.3 g and had increased to 281.9 ± 3.2 g at the time of sacrifice on Day 43. There were no significant differences between the groups at either Day 0 ($F = 0.358$; df = 4, 35; $p = 0.837$) or Day 43 ($F = 0.463$; df = 4, 35; $p = 0.763$). Five rats developed small fluid collections beneath their laminectomy incision, which subsequently resolved without complication. Two animals developed ischemic necrosis of the distal two-thirds of their tails immediately after the surgeries which likewise produced no further complication to their health or ability to perform on the various behavioral tasks. Two animals died after administration of the anesthetic and were therefore replaced with new rats to maintain 8 animals per group.

Injury parameters. A highly consistent injury was obtained across the groups. The recorded spinal cord displacements for all of the animals was 0.973 ± 0.003 mm; the groups were not different from each other ($F = 0.127$; df = 4, 35; $p = 0.972$). The overall recorded force of impact was 289.2 ± 5.2 kdyn, which was also not significantly different across the groups ($F = 1.905$; df = 4, 35; $p = 0.131$). One animal was immediately removed from the study (and replaced with a new animal) after the spinous process clamp slipped during the injury as indicated by grossly abnormal displacement and force curves.

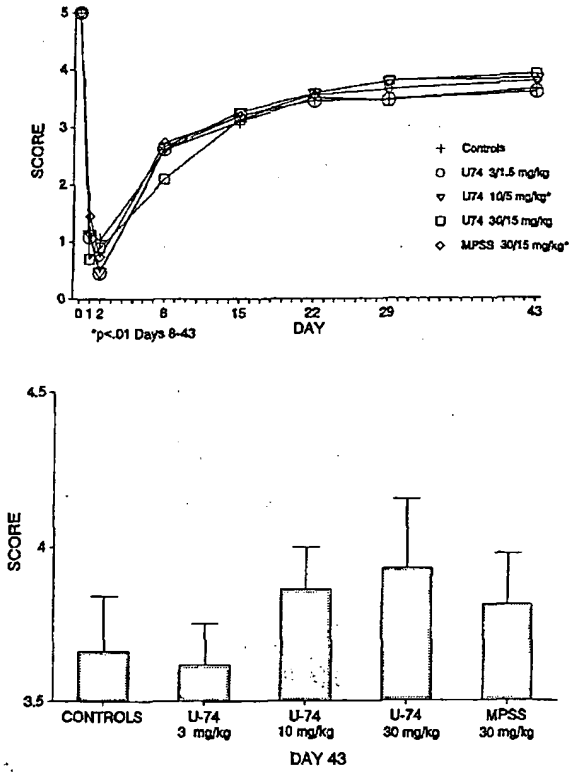

FIG. 2. U-74006F/MPSS open field walking scores. (Top) recovery curves indicate the mean scores of each group at each day tested (preinjury scores at Day 0). (Bottom) Day 43 group mean scores (histograms) and individual animal scores (dots). Initial bolus of the compounds (or equal volume vehicle) were given iv 5 min after the injury; these were followed with one-half the original dose at 2 and 6 h after the injury. (U74, U-74006F; MPSS, methylprednisolone sodium succinate.)

Behavioral results. Consistent injuries resulted in equally consistent behavioral scores as indicated in Fig. 2 (bottom) by the small range of open field walking scores recorded for the control animals on Day 43 (all scores between 3 and 4). These scores indicate that the majority of animals in this group recovered some coordinated walking ability. No significant differences were seen between the groups at Day 43 ($H = 2.043$; $p = 0.728$); however, each treatment group contained an animal(s) that recovered greater walking ability than the controls.

No significant differences in the behavioral scores were seen between the three different groups treated with U-74006F. However, examination of the open field walking scores over Days 8–43 (Fig. 2, top) demonstrated that the U-74006F (10 mg/kg initial bolus) and MPSS group scored significantly better than the controls, but did not differ from each other ($F = 5.287$;

TABLE 2

U-74006F/MPSS Day 43 Scores

	Open field walking (score)	Inclined plane (degrees)	Grid walking (footfalls/s)	Stride length (cm)	Toe spread (mm)	Front footprints (total–1 ft)	Base of support (cm)
Controls	3.66 ± 0.14	51.24 ± 0.88	1.21 ± 0.12	19.84 ± 0.65	17.09 ± 0.59	3.42 ± 0.24	3.29 ± 0.31
U74 (3/1.5 mg/kg)	3.62 ± 0.13	52.70 ± 0.77	1.24 ± 0.10	20.36 ± 0.74	16.92 ± 1.05	3.32 ± 0.11	2.95 ± 0.29
U74 (10/5 mg/kg)	3.86 ± 0.13	51.88 ± 1.16	1.14 ± 0.09	19.92 ± 0.35	17.02 ± 0.56	3.23 ± 0.11	3.78 ± 0.36
U74 (30/15 mg/kg)	3.93 ± 0.22	50.82 ± 0.84	1.34 ± 0.13	19.63 ± 0.45	16.30 ± 0.75	3.21 ± 0.13	3.00 ± 0.28
MPSS (30/15 mg/kg)	3.81 ± 0.16	51.45 ± 0.66	1.28 ± 0.06	19.53 ± 0.54	16.74 ± 0.49	3.18 ± 0.11	3.18 ± 0.40

df = 2, 21, 4; $p = 0.007$). Similar analysis of the inclined plane scores over Days 8–43 was not significant ($F = 0.160$; df = 2, 21, 4; $p = 0.852$).

No significant differences were found between any of the groups at Day 43 (Table 2) on the inclined plane ($F = 0.663$; df = 4, 35; $p = 0.622$), grid walking ($F = 0.558$, df = 4, 35; $p = 0.694$) or footprint measures: stride length ($F = 0.342$; df = 4, 34; $p = 0.847$), toe spread ($F = 0.195$; df = 4, 34; $p = 0.939$), number of front footprints/ft. walked ($F = 0.437$; df = 4, 35; $p = 0.781$), and base of support ($F = 1.038$; df = 4, 34; $p = 0.402$). Also, no group differences were noted at Day 29.

Histologic analysis. The lesions exhibited the same morphological features as previously described with this injury device (17, 30, 31) and with the weight-drop technique (20, 29, 33, 72). At the lesion center, there was extensive glial cell infiltration and severe central necrosis with areas of cyst formation; this was surrounded by a peripheral rim of spared tissue (demonstrated in Fig. 10, experiment 3). The calculated percentage of tissue spared at the lesion site (Fig. 3) for the control, U-74006F (10 mg/kg initial bolus), and MPSS groups were not significantly different ($F = 0.033$; df = 2, 21; $p = 0.968$).

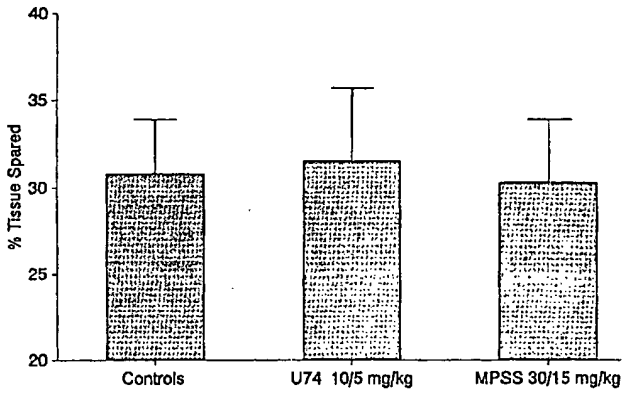

FIG. 3. U-74006F/MPSS percentage tissue spared at the lesion center (means ± SEM).

Experiment 2

Subjects. The group weights ($n = 80$, part 1 and part 2) were not significantly different at either the time of injury (overall mean 284.3 ± 2.9 g; $F = 0.090$; df = 6, 73; $p = 0.997$) or when they were sacrificed at Day 29 (overall mean 297.2 ± 4.4 g; $F = 0.160$, df = 6, 73; $p = 0.986$), although they showed a small weight gain over the course of the experiment. One death in this experiment resulted from the anesthetic administration. Two animals given a trial dose of MPSS 300/300/300/300 mg/kg also died of apparent respiratory complications within the first postoperative day. One rat developed a wound infection at the laminectomy site, and two others developed bladder infections; each infection was treated successfully with 7 days of antibiotics (Durapen, 20,000 U BID and Gentocin, 0.25 mg qD).

Injury parameters. Very consistent injuries were obtained in both part 1 and part 2 of this experiment. The mean recorded spinal cord displacement and impact force for all animals in part 1 was 1.105 ± 0.001 mm and 297.9 ± 4.6 kdyn; no group differences were observed for either the displacement ($F = 0.703$; df = 4, 34; $p = 0.595$) or force ($F = 0.927$, df = 4, 34; $p = 0.460$). In part 2, the mean recorded spinal cord displacement and force were 1.111 ± 0.001 mm and 314.0 ± 3.9 kdyn; again, no group differences were noted for either the displacement ($F = 0.317$, df = 4, 34; $p = 0.865$) or force ($F = 0.321$; df = 4, 34; $p = 0.862$).

Behavioral results. The Day 29 open field walking and inclined scores for animals ($n = 8$ per group) in part 1 are indicated in Table 3 (top). MPSS groups that received either the 30 mg/kg (×4) or 60 mg/kg (×4) doses scored consistently better than the other groups. Their scores were significantly better than the controls ($H = 6.955$, $p = 0.031$ Kruskall–Wallis ANOVA; $Z = 2.019$, $p = 0.044$ 30 mg/kg group, and $Z = 2.273$, $p = 0.023$ 60 mg/kg group, Mann–Whitney U test) but not different from each other ($Z = 0.621$, $p = 0.534$) in open field walking at Day 29 and over Days 8–29 ($F = 11.406$; df = 2, 21, 3; $p = 0.0001$). The 30 mg/kg (×4) and 60 mg/kg (×4) groups were not different from controls on inclined plane at Day 29 ($F = 2.961$; df = 2,

21; $p = 0.074$), but both scored higher over Days 8–29 ($F = 11.745$; df = 2, 21, 3; $p < 0.0001$).

Because the higher doses of MPSS were most effective in improving the animals behavioral recovery, part 2 of this experiment studied even higher doses (90 mg/kg ×4 and 120 mg/kg ×4) in conjunction with additional control, 30 mg/kg (×4) and 60 mg/kg (×4) animals ($n = 8$ per group). The Day 29 scores for these animals, indicated in Table 3 (bottom), demonstrate an apparent plateau effect above 30–60 mg/kg. No significant differences were found between the MPSS groups at Day 29 in open field walking ($H = 4.588$; $p = 0.205$) or on inclined plane ($F = 2.306$; df = 3, 28; $p = 0.098$); however, the 120 mg/kg (×4) group scored higher than the other MPSS groups in open field walking over Days 8–29 ($F = 3.889$; df = 3, 28, 3; $p = 0.012$), but this difference was not present on inclined plane ($F = 1.427$; df = 3, 28, 3; $p = 0.240$). An inverted U-shaped curve was not clearly observed in the current dosing range as previously reported by others using MPSS (52). Perhaps doses between 120 and 300 mg or extending the dosing interval out to 24–48 h would have resulted in similar findings.

Because the 30 mg/kg (×4) and 60 mg/kg (×4) groups of MPSS scored consistently better than the other groups, the open field walking scores (Fig. 4) and inclined plane scores (Fig. 5) for the control, 30 mg/kg (×4) and 60 mg/kg (×4) groups from part 1 and part 2 were combined ($n = 16$ per group). Both these MPSS groups had significantly better open field walking scores compared with the scores of controls ($H = 7.017$, $p = 0.030$, Kruskall–Wallis ANOVA; $Z = 2.441$, $p = 0.015$, 30 mg/kg group; and $Z = 1.982$, $p = 0.047$, 60 mg/kg group, Mann–Whitney U test) but not with each other ($Z = 0.532$, $p = 0.595$) at Day 29 and over Days 8–29 ($F = 6.763$; df = 2, 45, 3; $p = 0.002$). Both treatment groups also performed better on the inclined plane over Days 8–29 ($F = 9.399$; df = 2, 45, 3; $p = 0.0001$); only the 30 mg/kg group scored higher at Day 29 ($F = 4.040$; df = 2, 45; $p = 0.024$).

TABLE 3

MPSS Day 29 Scores

	Open field walking (score)	Inclined plane (degrees)
Part 1		
Controls	3.14 ± 0.09	54.39 ± 1.40
MPSS (30/15 mg/kg)	3.28 ± 0.07	56.02 ± 0.77
MPSS (30 mg/kg)	3.46 ± 0.08	57.28 ± 0.70
MPSS (45 mg/kg)	3.52 ± 0.17	55.41 ± 0.87
MPSS (60 mg/kg)	3.59 ± 0.13	57.50 ± 0.77
Part 2		
Controls	3.45 ± 0.14	57.09 ± 1.13
MPSS (30 mg/kg)	3.75 ± 0.10	60.21 ± 0.50
MPSS (60 mg/kg)	3.51 ± 0.12	57.09 ± 1.21
MPSS (90 mg/kg)	3.45 ± 0.10	56.25 ± 1.47
MPSS (120 mg/kg)	3.81 ± 0.19	57.92 ± 1.08

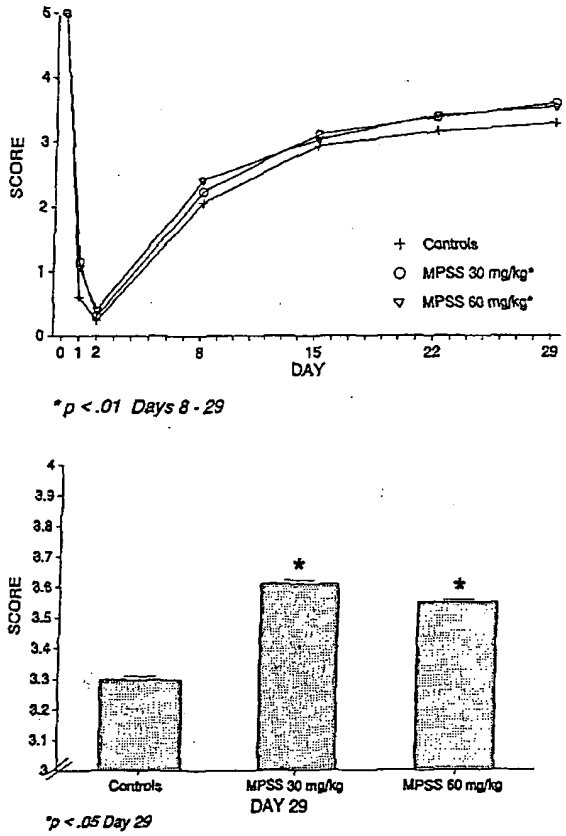

FIG. 4. MPSS open field walking scores. (Top) recovery curves indicate the mean scores of each group at each day tested. (Bottom) Day 29 group mean scores ± SEM. MPSS was administered iv bolus 5 min, 2, 4, and 6 h after the injury.

Histologic analysis. Determination of the percentage of spared tissue at the lesion center (Fig. 6) was performed for only the control, 30 mg/kg (×4), and 60 mg/kg (×4) MPSS groups ($n = 16$ per group). Only the 60 mg/kg (×4) group had significantly greater tissue sparing than the controls ($F = 3.228$, df = 2, 42; $p = 0.049$); the two MPSS groups were not significantly different.

Power analysis. Results of the power analysis (Table 4) indicated that groups of approximately 15 animals are needed to reproduce the MPSS effects with 90% predictability. These predictions are dependent upon identical experimental conditions (e.g., injury severity, animal size/age, etc.); therefore, these numbers provided only an estimate for designing the subsequent experimental protocol.

Experiment 3

Subjects. As previously described, the animals treated immediately postinjury with YM-14673 awakened much

sooner and exhibited increased spontaneous motor activity (oral and limb) compared with the other animals (79, 93, 94). However, consistent differences between the animals could not be identified by the examiners (unaware of the study groups) during the postinjury testing periods. Comparison of the test scores of all animals at Day 29 before and after administration of the YM-14673 (1 mg/kg) revealed no statistically significant differences on open field walking ($F = 0.018$; df = 6, 1; $p = 0.894$) or grid walking ($F = 0.079$; df = 6, 1; $p = 0.782$). The inclined plane scores (Day 29) were significantly lower after administration of the YM-14673 ($F = 4.030$; df = 6, 1; $p = 0.046$). However, each of the study groups showed a similar decrease in their scores. This could reflect an artifact of the testing sequence; in addition, there could be a specific drug effect that results in the YM-14673 groups scoring lower. As a result, the scores for all of the animals were averaged over the two testing times (before and after YM-14673 administration) to control for these effects.

The overall mean weight of the animals ($n = 105$) at the time of injury (Day 0) was 284.6 ± 1.8 g, which had increased to 294.4 ± 2.0 g by Day 29. The groups were not different from one another at Day 0 ($F = 0.805$; df = 6, 98; $p = 0.569$). However, at Day 29, the animals treated with YM-14673 (1/1/1/1 mg/kg iv and 1 mg/kg/day ip) were significantly heavier (mean weight 317.5 ± 6.7 g) than the other groups ($F = 4.689$; df = 6, 98; $p = 0.0003$).

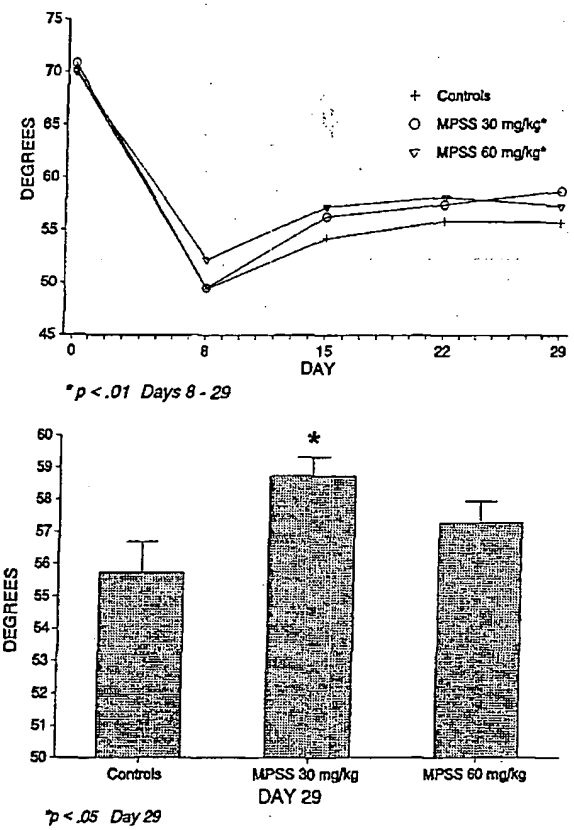

FIG. 5. MPSS inclined plane scores. (Top) recovery curves indicate the mean scores of each group at each day tested. (Bottom) Day 29 group mean scores ± SEM. MPSS was administered iv bolus 5 min, 2, 4, and 6 h after the injury.

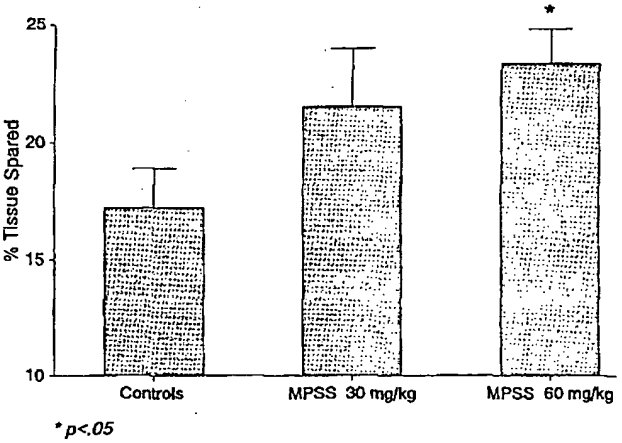

FIG. 6. MPSS percentage tissue spared at the lesion center (means ± SEM).

Despite the very large number of animals involved in this experiment ($n = 107$), few complications were observed. One death occurred in the immediate postoperative period from unknown causes. Two animals developed intraabdominal tumors; one reached massive size (5 cm) and necessitated the removal of this animal from the study. Four animals developed small fluid collections under the laminectomy incision as in experiment 1 which resolved spontaneously. One urinary tract infection and a laceration on the back of one animal (caused by its cagemate) were treated successfully with 7 days of antibiotics (Durapen, 20,000 U BID and Gentocin, 0.25 mg qD).

Injury parameters. Similar consistency in the recorded spinal cord displacements (1.119 ± 0.002 mm) and impact forces (299.7 ± 2.8 kdyn) was seen in this experiment. There were no significant group differences for either displacement ($F = 0.333$; df = 6, 97; $p = 0.918$) or force ($F = 0.113$; df = 6, 97; $p = 0.995$).

TABLE 4

Methylprednisolone Power Analysis

	Δ Score	Standard deviation	$p = 0.9$	$p = 0.7$
Open field walking	0.28	0.33	$n = 15$	$n = 9$
Inclined plane	2.28	2.99	$n = 19$	$n = 11$
% Tissue spared	5.20	6.85	$n = 15$	$n = 9$

Behavioral results. The Day 29 scores for each of the study groups are indicated in Table 5. MPSS, when given as an initial bolus of 60 mg/kg followed by 30 mg/kg per dose (×3), resulted in greater improvement in that group's open field walking scores over Days 8–29 compared to the group treated with 30 mg/kg (×4) ($F = 7.109$; df = 1, 28, 3; $p = 0.009$); no other significant differences were noted between the two groups.

The YM-14673 group treated both acutely and chronically (1/1/1/1 mg/kg iv) and 1 mg/kg/day ip Days 1–29) demonstrated consistently better behavioral scores (not statistically significant) than either of the groups treated only acutely (1/1/1/1 mg/kg iv) or chronically (1 mg/kg ip Days 15–29); therefore, the acute/chronic group was used for all statistical comparisons with the MPSS group. It is important to note, however, that the YM-14673 group treated only chronically had significantly higher open field walking scores over Days 8–29 ($F = 4.745$; df = 2, 42, 3; $p = 0.010$) compared with the controls. Similarly, the YM-14673 animals treated only acutely scored higher than the control animals on the inclined plane over Days 8–29 ($F = 7.547$; df = 2, 42, 3; $p = 0.0008$). Therefore, both acute and chronic therapy with YM-14673 improved performance, but the combination of acute and chronic therapy was even more efficacious.

Comparison of the control animals with the MPSS group (60/30/30/30 mg/kg) and the acute/chronic YM-14673 group (1/1/1/1 mg/kg iv and 1 mg/kg/day ip) in open field walking (Fig. 7) showed the YM-14673 animals to perform significantly better than both the MPSS and control animals over Days 8–29 ($F = 8.755$; df = 2, 42, 3; $p = 0.0003$); no differences were seen between the groups at Day 29 ($F = 0.302$, df = 2, 42; $p = 0.741$). The relatively lighter injury compared with that in experiment 2 (from recalibration of the injury device power amplifier) may account for the lack of a significant open field walking effect at Day 29. Both the MPSS and the YM-14673 groups scored significantly better than the control animals on the inclined plane (Fig. 8) over Days 8–29 ($F = 6.353$; df = 2, 42, 3; $p = 0.002$); only the YM-14673 animals were better at Day 29 ($F = 3.303$; df = 2, 42; $p = 0.046$). Results of the grid walking (Fig. 9) at Day 29 also demonstrated significant improvement for the YM-14673-treated animals compared to the controls ($F = 3.623$; df = 2, 41; $p = 0.036$).

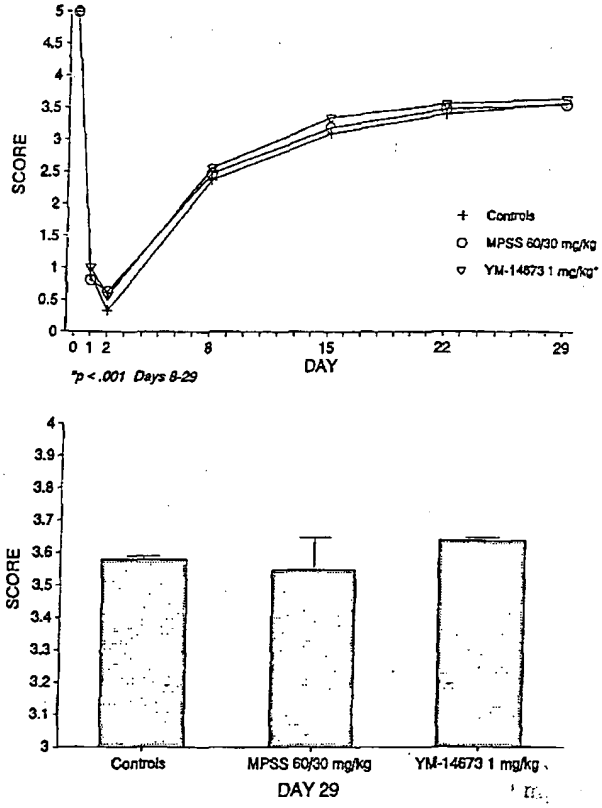

FIG. 7. YM-14673/MPSS open field walking scores. (Top) recovery curves indicate the mean scores of each group at each day tested. (Bottom) Day 29 group mean scores ± SEM. YM-14673 (1 mg/kg) and MPSS (60/30/30/30 mg/kg) were administered iv bolus 5 min, 2, 4, and 6 h after the injury; YM-14673 animals also received 1 mg/kg/day over Days 1–29. (YM, YM-14673.)

TABLE 5

YM-14673/MPSS Day 29 Scores

	Open field walking (score)	Inclined plane (degrees)	Grid walking (footfalls/s)
Controls	3.58 ± 0.07	54.72 ± 0.83	1.42 ± 0.05
MPSS (30 mg/kg)	3.54 ± 0.08	56.22 ± 0.79	1.42 ± 0.06
MPSS (60/30 mg/kg)	3.55 ± 0.10	56.61 ± 0.51	1.38 ± 0.06
YM Acute (1 mg/kg)	3.71 ± 0.10	56.34 ± 0.70	1.26 ± 0.06
YM Acute and chronic (1 mg/kg)	3.64 ± 0.09	57.05 ± 0.66	1.24 ± 0.05
YM Chronic (1 mg/kg)	3.61 ± 0.11	56.22 ± 0.71	1.39 ± 0.06
YM (1 mg/kg) and MPSS (30 mg/kg)	3.64 ± 0.09	56.50 ± 0.57	1.37 ± 0.07

No significant additive effect was seen with the combination of MPSS (30 mg/kg ×4) and YM-14673 (1 mg/kg ×4). The combination of the two drugs resulted in the same score as either individually, or, in the case of open field walking, the combination-treated animals and the YM-14673-treated animals scored significantly higher over Days 8–29 than those animals receiving MPSS ($F = 5.715$; df = 2, 42, 3; $p = 0.004$).

Histologic analysis. Because the MPSS (60/30/30/30 mg/kg) and acute/chronic YM-14673 (1/1/1/1 mg/kg iv plus 1 mg/kg/day ip) groups scored consistently the best on the behavioral measures, only these treatment groups and the controls underwent histologic analysis. As in experiments 1 and 2, the gross morphology of the lesions demonstrated extensive cyst forma-

tion and glial cell infiltration at the lesion centers; a small peripheral rim of white matter remained as seen in Fig. 10. Essentially no sparing of gray matter was seen at this injury level for any of the study groups. The results demonstrated that both treatment groups had significantly greater tissue sparing at the lesion center (Fig. 11) than the controls ($F = 8.390$; df = 2, 42; $p = 0.0009$).

DISCUSSION

Summary

This is the first report of significant behavioral and histologic effects in rats after treatment with MPSS subsequent to a spinal cord contusion injury. Treatment of the animals with U-74006F resulted in some improvement in behavioral recovery that was equivalent to the effects seen after treatment with a lower, less effective dose of MPSS. Administration of YM-14673 resulted in significantly better behavioral and histologic scores than those of control animals; YM-14673 animals also had consistently better behavioral scores than MPSS-treated animals, indicating that YM-14673 may be beneficial after clinical spinal cord injury.

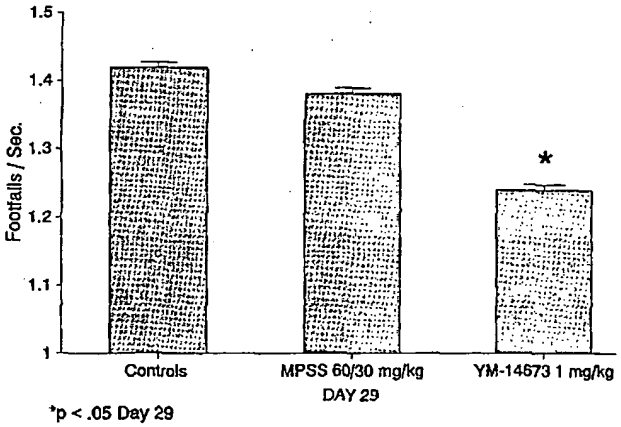

FIG. 9. YM-14673/MPSS grid walking scores. Day 29 group mean scores ± SEM. YM-14673 (1 mg/kg) and MPSS (60/30/30/30 mg/kg) were administered iv bolus 5 min, 2, 4, and 6 h after the injury; YM-14673 animals also received 1 mg/kg/day over Days 1–29.

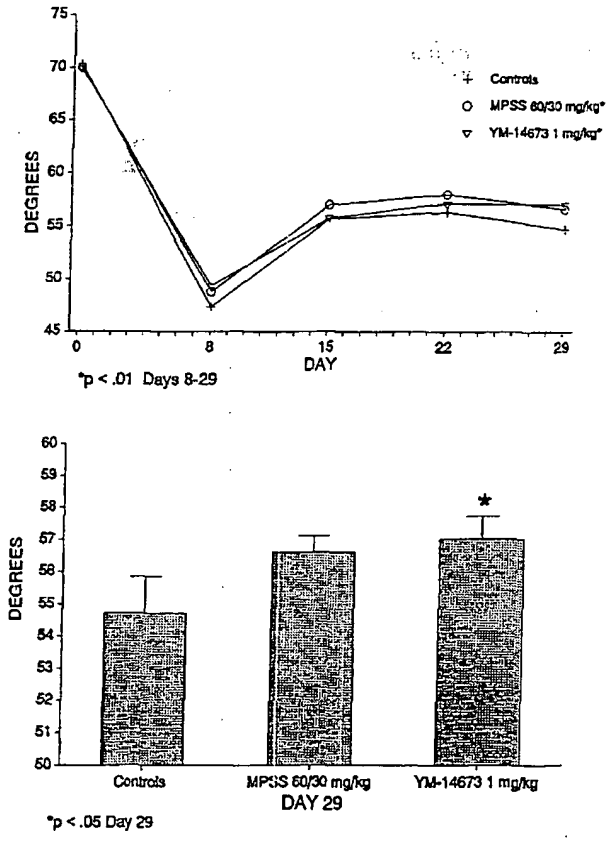

FIG. 8. YM-14673/MPSS inclined plane scores. (Top) recovery curves indicate the mean scores of each group at each day tested. (Bottom) Day 29 group mean scores ± SEM. YM-14673 (1 mg/kg) and MPSS (60/30/30/30 mg/kg) were administered iv bolus 5 min, 2, 4, and 6 h after the injury; YM-14673 animals also received 1 mg/kg/day over Days 1–29.

Injury Model

These data support the usefulness of this model for preclinical testing of pharmacological agents. Small variability within the groups results in the ability to detect relatively small changes in behavior and lesion size without the need for large numbers of animals. The low interanimal variability results from a highly reproducible displacement of the spinal cord (10, 17, 31, 73, 74, 80, 82). Biological variability (for example, differences in the size of the animals spinal cords) may explain the relatively larger variability in the force required to produce an equivalent displacement (19).

When comparing the results of these three sequential experiments, slight drift was noted in the severity of the injury and the resulting behavioral and histologic outcome. Recalibration of the injury device power amplifier as well as other technical problems may account for these differences. As a result of these technical differences and the inherent differences in the experimental protocols, only general conclusions can be made between the experiments.

Functional outcome in this model is readily quantified by relatively simple behavioral measures (9, 17, 31). Independent application of these behavioral tests allows us to identify variable drug effects at different levels of injury severity. For example, the more severe injury in experiment 2 resulted in the least recovery of gross locomotor function in open field walking; MPSS administration in this experiment resulted in animals with

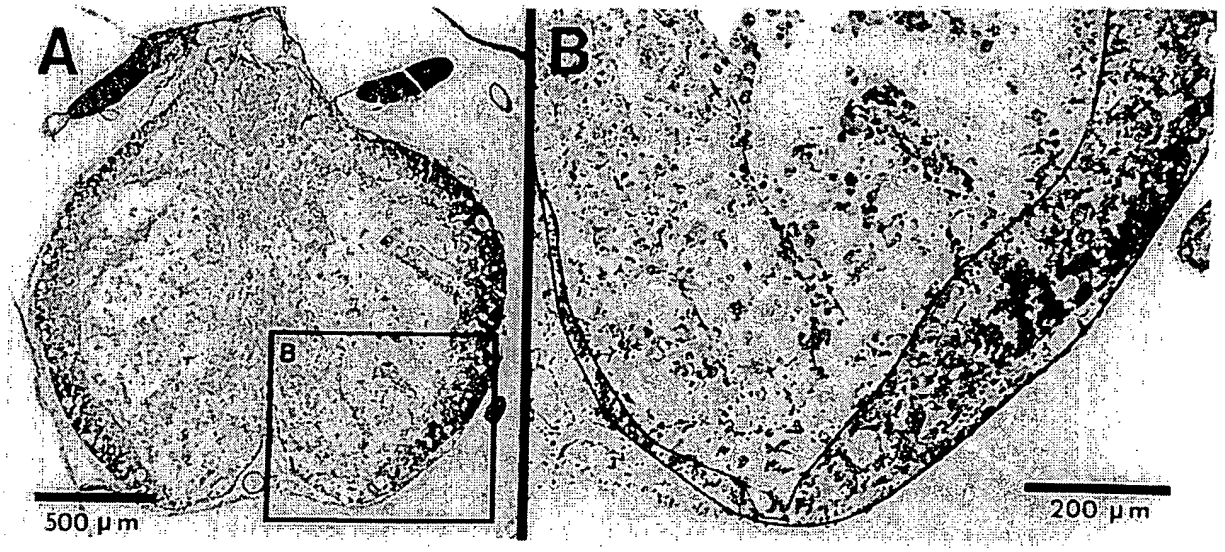

FIG. 10. Cresyl violet and luxol fast blue stained paraffin section of the lesion center from the median animal in the control group. (A) Low-power magnification (4×) demonstrates significant cystic necrosis of the cord and sparing of white matter at the periphery. (B) Higher-power magnification (10×) of the ventral-lateral funiculus (the black line demarcates the region of spared white matter).

significantly better open field walking scores compared to the controls. In experiment 3, however, a relatively less severe injury resulted in essentially all of the animals recovering coordinated gross locomotion; no significant improvement was noted in open field walking in this experiment for the animals treated with MPSS compared with controls. The grid walking test, which may be influenced by sparing of propriospinal systems in the cord (31, 65), was able to detect small residual deficits in hindlimb function (experiment 3) that were not observable in open field walking. Only YM-14673-treated animals scored significantly better than the controls on grid walking. The current use of composite scoring regimens (76, 91) designed to enhance sensitivity prevents detection of these individual differences (42). Furthermore, averaging of both positive and potentially negative treatment effects may result in incorrect conclusions.

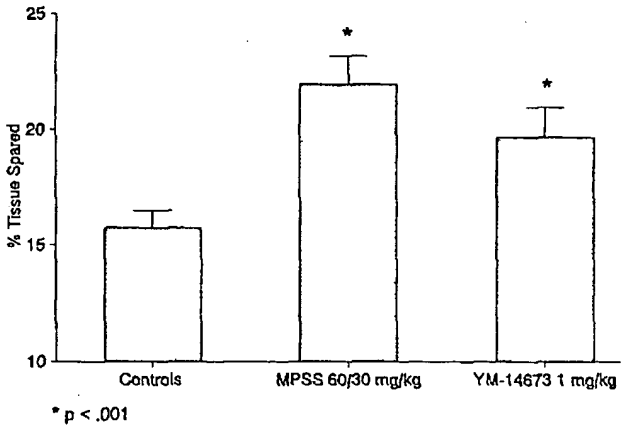

FIG. 11. YM-14673/MPSS percentage tissue spared at the lesion center (means ± SEM).

Contusion injuries that result in an incomplete neurologic deficit may provide the best approximation of clinical spinal cord injury (34, 62, 63, 89). However, contusion injury alone does not replicate the entire spectrum of clinical spinal cord injuries, e.g., compression vs contusion of the spinal cord (4, 19). Different mechanisms of injury, in addition to differences in injury severity, animal models, and drug delivery/dosing, may significantly influence the response observed after administration of a given compound. These factors may account for the variable behavioral effects observed with MPSS treatment in this study and by other investigators after experimental spinal cord injury (26, 36, 43, 59, 70).

Pharmacologic Studies

The primary mechanism of action of MPSS after acute spinal cord injury has been reported to involve inhibition of oxygen free radical-mediated lipid peroxidation (for review, see Hall, 1992) (46). Additional and possibly related actions include preservation of spinal cord blood flow (50, 59, 104), attenuation of intracellular calcium accumulation (104), reduction of neurofilament degradation and improvement in energy metabolism (25), and inhibition of inflammatory processes (60). U-74006F, a 21-aminosteroid that lacks glucocorticoid activity, has been demonstrated to have even greater ability than MPSS to inhibit lipid peroxidation (27) (for

review, see Hall *et al.*, 1992 (52) and Hall *et al.*, 1988) (47); U-74006F has also been demonstrated to attenuate excitotoxic neuronal injury in cortical cell cultures (71).

U-74006F has been shown to protect against the acute effects of brain injury (49, 54, 68) and spinal cord ischemia (41, 44, 53); the effects in brain ischemia have been mixed (11, 48, 51, 55, 75). This compound has also been shown to improve neurologic recovery after experimental spinal cord injury in cats (1, 2, 47) and in rats (58). In each of these latter studies, spinal cord injury was made by compressing the cord for a variable period of time. In the present study, the different method of injury (contusion), as well as the relatively less severe injury used may account for the small behavioral improvement observed after administration of this compound. In addition, increased variability in the control group compared with that in experiments 2 and 3 may have limited our ability to detect small behavioral improvement. Further dose–response studies of U-74006F are necessary to determine its maximal effect in this model.

YM-14673 is a TRH analogue which maintains the integrity of the C-terminal amino acid critical for the neuroprotective effects of TRH analogues (38). Several proposed actions of TRH (and TRH analogues) that may relate to the neuroprotective effects include the ability to reverse or attenuate certain physiological effects of opioids (57, 97), to improve tissue blood flow (37, 64), and to attenuate the hypotensive effects of leukotriene D4 (66) and platelet-activating factor (67). The TRH analogue, CG3703, has been reported to restore magnesium homeostasis (87) and intracellular brain metabolism after fluid-percussion brain injury (69), and to attenuate changes in several tissue cations and improve energy metabolism following experimental spinal cord injury (39). TRH has also been demonstrated to exert neurotrophic effects on cultured ventral horn neurons (5, 7, 61, 78), lower motoneurons *in vivo* (6, 40, 86, 88), and spinal cord transplants in oculo (56). In this study, the improved behavioral recovery observed in animals treated chronically with YM-14673 (Days 15–29) suggests that YM-14673 may also have neurotrophic effects and possibly influence spinal cord plasticity and reorganization after contusion injury (8, 10, 81). In addition, the reported central stimulatory and increased locomotor effects of YM-14673 (79, 93, 94) may result in improved behavioral scores through a generalized facilitation of motor neuron firing.

YM-14673 has been shown to exert neuroprotective effects in several different models of central nervous system injury, including experimental cerebral hematoma (95), focal cerebral ischemia (96, 100, 101), stroke-prone spontaneously hypertensive rats (99), and electrical lesion of the internal capsule (98). YM-14673 has also been shown to improve neurologic outcome in models of brain injury (35) and spinal cord injury (35, 76). In a previous study using this injury device, treatment with YM-14673 had a significant effect on behavioral recovery and histologic sparing for animals treated with a single intravenous dose (1 mg/kg) 30 min after the injury (15, 16). In that study, significant improvement was observed in both open field walking and inclined plane; the injury produced was relatively more severe (comparable to that obtained in experiment 2 of this study) such that assessment of performance on the grid walking task was not possible due to insufficient recovery of locomotor ability.

YM-14673 administration in this study after a relatively lighter injury also resulted in significantly better behavioral and histologic effects than controls. In addition, YM-14673 demonstrated greater efficacy than MPSS under these experimental conditions. A YM-14673 response was seen after both acute and chronic administration, and YM-14673 demonstrated the greatest effects with combined acute and chronic treatment. These data indicate that YM-14673 may be beneficial in treatment of clinical spinal cord injury, and that there may be multiple mechanisms of action of this drug.

Preclinical Trials

It seems likely that a multitude of compounds with multiple neuroprotective, trophic, and central nervous system stimulatory effects will be even more efficacious than the currently established postinjury treatments with MPSS. The availability of standardized protocols for the evaluation of acute and chronic effects of drugs on recovery of function in the rat should provide a means for preclinical evaluations of such compounds and their potential additive effects.

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